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Research Note

2-Dodecylcyclobutanone Does Not Induce Mutations in the *Salmonella* Mutagenicity Test or Intrachromosomal Recombination in *Saccharomyces cerevisiae*†

CHRISTOPHER H. SOMMERS[1]* AND ROBERT H. SCHIESTL[2]

[1]*U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038;* [2]*Departments of Pathology, Environmental Health and Radiation Oncology, David Geffen School of Medicine and School of Public Health, UCLA, 650 Charles E. Young Drive South, Los Angeles, California 90024, USA*

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ABSTRACT

Treatment of foods, such as red meat and poultry, that contain palmitic acid with ionizing radiation leads to the formation of 2-dodecylcyclobutanone (2-DCB), a compound found only in irradiated foods. In this study, the *Salmonella* mutagenicity test and the yeast DEL assay were used to evaluate the genotoxic potential of 2-DCB. *Salmonella* Typhimurium tester strains TA98, TA100, TA1535, and TA1537 were exposed to 0, 0.125, 0.25, 0.5, and 1 mg per well of 2-DCB, with and without exogenous metabolic activation (5% S9 fraction), using the microtiter plate–based Miniscreen version of the test. 2-DCB did not induce mutations in the *Salmonella* mutagenicity test. When *Saccharomyces cerevisiae* strain RS112, which contains a nonfunctional duplication of the *his3* gene that can be induced to form a functional *HIS3*$^+$ gene by intrachromosomal recombination, was exposed to 0.63, 1.25, 2.5, or 5.0 mg/ml of 2-DCB, no increase in the rate of intrachromosomal (DEL) recombination was observed. The absence of genotoxicity observed in this study using purified 2-DCB agrees with the lack of genotoxic and teratogenic activity observed in previously conducted multigeneration feeding studies of laboratory animals (rats, mice, guinea pigs, and rabbits) that used radiation-sterilized poultry that contained 2-DCB as a unique radiolytic product.

Exposure of foods that contain fatty acids to ionizing radiation leads to the formation of a class of compounds known as the alkylcyclobutanones, which are not detectable in nonirradiated food products *(7, 12, 19, 29).* Cleavage of the acyl-oxygen bonds of palmitic acid by ionizing radiation can lead to its cyclization, resulting in a molecule with the same number of carbon atoms as palmitic acid with an alkyl group in the second ring position, or 2-dodecylcyclobutanone (2-DCB) *(19).* 2-DCB ($C_{16}H_{30}O$; FW, 238.41) is produced in trace quantities (approximately 0.1 μg of 2-DCB per g of fat) in irradiated foods such as red meat and poultry *(7, 12, 19, 29).*

Many types of short-term genetic toxicology tests have been used to evaluate the genotoxic potential of food additives and chemicals formed by processing technologies. One of these tests, the comet assay, is designed to detect DNA strand breaks in bacterial, fungal, or mammalian cells via an increase in the electrophoretic mobility of smaller DNA fragments that are formed as a result of DNA damage and chromosome fragmentation *(31).* Recently, studies that used the comet assay claimed that 2-DCB induced DNA strand breaks in rodent and human intestinal cells, without exogenous metabolic activation, which raised the possibility that the compound was a weak genotoxin *(13, 14).* The work received a great deal of attention in the United States due to introduction of irradiated ground beef into the U.S. National School Lunch Program beginning in 2004, with some consumer groups erroneously claiming that results using the comet assay showed that 2-DCB was mutagenic and that irradiated foods therefore caused cancer *(3, 4).* In contrast, review of those results *(13, 14)* by international regulatory agencies indicated that the interpretation that 2-DCB was genotoxic could not be supported based on the data and methods used *(16, 18).* The comet assay, although it is used extensively as a screening assay, has not been validated for the detection of weak genotoxins and can produce false-positive results due to the chromosome degradation that occurs as a result of nongenotoxic cell death *(18, 31).*

To more accurately assess the potential genotoxicity of 2-DCB, its ability to induce mutations in the *Salmonella* mutagenicity test and genomic rearrangements in the yeast DEL assay was determined. The *Salmonella* mutagenicity test has been used for more than 25 years as a reliable, short-term genetic toxicology test *(2, 20). Salmonella* Typhimurium tester strains TA98, TA100, TA1535, and TA1537 (Table 1) detect reversion of mutations in genes required for histidine synthesis, resulting in conversion of cells from histidine auxotrophy to histidine prototrophy. The *Salmonella* Typhimurium tester strains used in the test have been genetically engineered to have decreased nucle-

* Author for correspondence. Tel: 215-836-3754; Fax: 215-233-6445; E-mail: csommers@arserrc.gov.

† Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

TABLE 1. *Genotypes of the* Salmonella *Typhimurium Tester Strains*

Strain	Lesion bypass[a]	DNA repair[b]	Permeability[c]	Mutation	Detects
TA98	pKM101	Δ*uvrB*	*rfa*	*hisD3052*	Framshift mutations
TA1537	No	Δ*uvrB*	*rfa*	*hisC3076*	Frameshift mutations
TA100	pKM101	Δ*uvrB*	*rfa*	*hisG46*	Point mutations
TA1535	No	Δ*uvrB*	*rfa*	*hisG46*	Point mutations

[a] The plasmid pKM101 carries the *mucAB* genes, which are homologs of the *Escherichia coli* and *Salmonella umuDC* genes required for trans-lesion DNA synthesis, and increased sensitivity of the tester strains to mutagens *(2).*

[b] The *uvrB* gene enhances detection of mutagens through elimination of nucleotide excision repair *(2).*

[c] The *rfa* mutation leads to a defective lipopolysaccharide layer, allowing permeability of the tester strains to large molecules *(2).*

otide excision repair activity and cell walls that are more permeable to large-molecular-weight compounds to increase their sensitivity to genotoxins (Table 1) *(2).* The *Salmonella* strains were exposed to 2-DCB, with and without exogenous metabolic activation, using the microtiter plate–based Miniscreen version of the plate incorporation test *(2, 8, 10, 15).*

The yeast (*Saccharomyces cerevisiae*) DEL assay measures a compound's ability to cause genomic rearrangements, induced by DNA strand breakage, by restoration of a nonfunctional duplication of the *his3* gene to functionality (*HIS3*$^+$) by intrachromosomal (DEL) recombination. (Fig. 1) *(22).* The assay does not produce false-positive results due to cell death, because only recombination events in live cells are selected for and quantified. The yeast DEL assay detects many carcinogens that are positive, as well as carcinogens that are negative, in the *Salmonella* mutagenicity test *(9, 22, 24, 26).* Furthermore, both mutagenic and nonmutagenic carcinogens induce DEL recombination between two copies of a nonfunctional hypoxanthene (guanine)

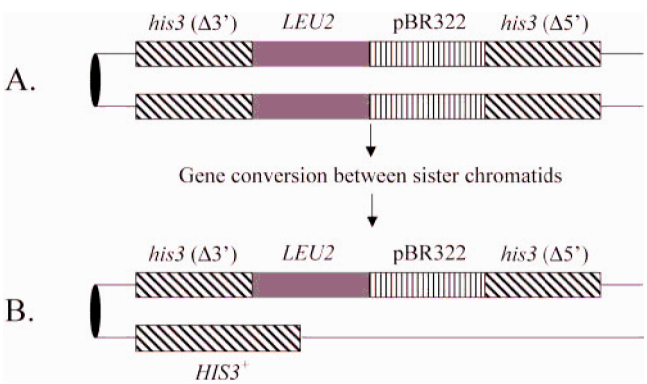

FIGURE 1. *(A) Yeast strain RS112 contains a duplication of the* his3 *gene in which one of the alleles is deleted at its 3′ end and the other for its 5′ end, with intervening* LEU2 *gene and pBR322 plasmid DNA. The two alleles share approximately 400 bp of homology and thus can recombine with each other to form a* HIS3$^+$ *allele. (B) Following DNA strand breakage in the region of the* his3 *gene duplication by a genotoxin or the DNA repair process, gene conversion between sister chromatids results in one chromatid that remains* his3 LEU2$^+$ *and the other* HIS3$^+$ *leu2*$^-$ *(DEL recombination). Chromosome segregation and cell division then results in one* S. cerevisiae *cell that carries the recombined* HIS3$^+$ *chromosome (22).*

phosphoribosyltransferase gene duplication in vitro in human cells *(5)* and in vivo between two copies of a nonfunctional gene duplication in pinkeye unstable C57BL/6J mice *(23).* The ability of 2-DCB to induce mutations and chromosomal rearrangements in the *Salmonella* mutagenicity test and yeast DEL assay are presented and discussed.

MATERIALS AND METHODS

***Salmonella* mutagenicity test: strains.** *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 were purchased from Moltox Inc. (Boone, N.C.) or from Xenometrix Inc. (Boulder, Colo.). The strains were tested for histidine dependence, ampicillin resistance, UV sensitivity, crystal violet sensitivity, and spontaneous reversion frequency *(2, 20).* The tester strains were propagated on Vogel-Bonner minimal medium supplemented with 25 μg/ml of histidine and 0.05 mM D-biotin, with or without 25 μg/ml of ampicillin, and stored at 0 to 2°C for up to 1 week before use in assays *(2).* For use in the assays, the tester strains were grown, from single colonies, in 100 ml of sterile nutrient broth in 500 ml of baffled Erlenmeyer flasks (37°C, 150 rpm) for approximately 16 h.

Media and media components. Nutrient broth was obtained from Difco Inc. (Sparks, Md.). Vogel-Bonner salts were obtained from Moltox Inc. Sodium chloride, glucose, D-biotin, and L-histidine HCl were obtained from Sigma-Aldrich Inc. (St. Louis, Mo.). Sterile six-well microtiter plates (35-mm-diameter wells) (Corning Inc., Corning, N.Y.) were prepared by dispensing 5 ml of sterile minimal agar into each well *(2).* Sterile top agar was melted using a microwave oven, cooled to 45°C in a heated water bath, and filter sterilized with histidine and biotin solution added to a final concentration of 0.05 mM *(2).*

Chemicals. The positive control compound methyl methanesulfonate (MMS) (CAS no. 66-27-3) was obtained from Sigma-Aldrich, Inc. The positive control compounds 2-nitroflourene (2-NF) and 2-aminoanthracene (2-AA) (CAS no. 613-13-8) were obtained from Moltox Inc. 2-DCB (CAS no. 35493-46-0), analytical testing grade (>95%) for detection of irradiated foods by regulatory agencies, was obtained from Sigma-Aldrich Inc. 2-DCB was suspended in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Inc.) as previously described by Delincee et al. *(13, 14),* as were the MMS, 2-NF, and 2-AA positive control compounds. 2-DCB concentrations used in the assay were 1.0, 0.5, 0.1, and 0.05 mg per well. MMS was used at 120 μg per well, 2-AA at 10 μg per well, and 2-NF at 10 μg per well.

Exogenous metabolic activation. S9 fraction from Aroclor 1254 induced rats was obtained from Moltox Inc., as was NADPH

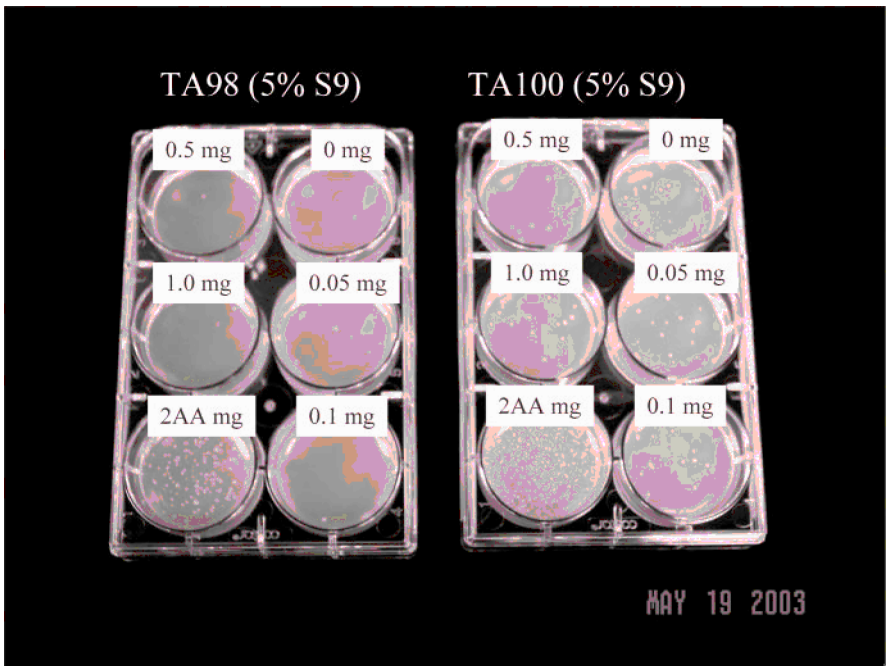

FIGURE 2. *Microtiter plate setup for testing of 2-dodecylcyclobutanone (2-DCB) in the* Salmonella *mutagenicity test. Two test plates were used per* Salmonella *culture (TA100, TA1535, TA98, and TA1537) tested. Each experiment was conducted independently three times, with or without exogenous metabolic activation (5% S9 fraction). Test compound concentrations included the negative (solvent) control, 0.05 mg of 2-DCB, 0.1 mg of 2-DCB, 0.5 mg and 1.0 mg of 2-DCB, and positive control compounds (MMS, 2-NF, or 2-AA).*

regeneration system components A and B. S9 fraction (5% solution) was prepared immediately before performance of the assays *(2, 10).* S9 fraction solution was maintained on ice during the assay procedure.

Assay procedure. Due to the expense of 2-DCB (>$12,000 per gram), the microtiter plate–based Miniscreen assay *(8, 15),* which uses 20% of the test compound, cells, solvent, and S9 fraction of the standard plate incorporation assay, was used. In short, 500 μl of top agar (45°C), 20 μl of test compound (solvent alone, solvent with 2-DCB, or solvent with positive control compound), 20 μl of overnight culture, and 100 μl of 5% S9 solution (if required) were combined, mixed by vortexing, and dispensed into the well of a microtiter plate that contained 5 ml of minimal agar. After allowing 1 h for solidification of the top agar, the plates were incubated at 37°C for 2 days and the colonies per well scored using an AccuCount 1000 calibrated colony counter (AccuCount Inc., Gainesville, Va.). Cytotoxicity was determined by examination of the bacterial lawn in the top agar, following the 2-day incubation *(2, 10),* which is standard for the bacterial reverse mutation assays. Microtiter plate setup is shown in Figure 2.

The yeast DEL assay: yeast propagation. *S. cerevisiae* strain RS112 (*MAT***a**/α *ura3-52/ura3-52 leu2-3,112/leu2*Δ*98 trp5-27/TRP5 arg4-3/ARG4 ade2-40/ade2-101 ilv1-92/ILV1 HIS3*:: *pRS6/his3*Δ*200 LYS2/lys2-801*) was obtained from Dr. Robert Schiestl (UCLA, Los Angeles, Calif.). All yeast media components were purchased from Sigma-Aldrich. The media was prepared and the assay was performed as described by Schiestl *(22). S. cerevisiae* RS112 was pregrown in 25 ml of leucine omission medium (LOM) to stationary phase (30°C, 18 h, 200 rpm) in a sterile, 50-ml culture tube (Corning, Inc.) in a shaking incubator. The LOM prevents the growth of yeast cells that develop intrachromosomal recombination events, because the medium selects for leucine prototrophs those that retain the recombination system during the culture growth.

Exposure to chemicals. 2-DCB and MMS were suspended in DMSO as previously described. *S. cerevisiae* RS112 was diluted to a cell density of 1 to 2 × 10^6 cells per ml of which 5 ml was divided into 50-ml sterile culture tubes. DMSO (100 μl) was added to the negative control sample (2%, vol/vol). 2-DCB suspended in 100 μl of DMSO was added to the 5-ml aliquots to obtain final concentrations of 0.63, 1.25, 2.5, and 5.0 mg/ml. MMS was added to a final concentration of 50 μg/ml. RS112 was then allowed to grow in the presence of the test compound for 18 h (30°C, 200 rpm) in a shaking incubator. *S. cerevisiae* does not require exogenous metabolic activation due to its internal enzymatic activity *(11).* Cells that undergo recombination (Fig. 1) to form the *HIS3*$^+$ allele progress to stationary phase during the overnight incubation but do not proliferate further. Therefore, in this system, the recombination frequency is indicative of the recombination rate.

Determination of recombination frequency. Following the exposure to test compound, the cells were spun down and the pellet was resuspended in an equal volume of Butterfield's phosphate buffer and diluted serially (1/10) in Butterfield's phosphate buffer. Aliquots of the diluted yeast (100 μl) were then surface plated, in triplicate, onto synthetic complete medium agar to assess cell viability and histidine omission medium agar to select for recombinants. The agar plates were then incubated at 30°C for 2 days, the plates for CFU scored using an AccuCount 1000 calibrated colony counter, and the recombination frequencies and percentage of viable cells determined *(22).*

Statistical analysis. For the *Salmonella* mutagenicity test, two replicate plates were used per bacterial tester strain culture. Each experiment was performed independently three times. For the yeast DEL assay, each experiment was conducted independently three times. The Student's *t* test, using the statistics package of Microsoft Excel (Microsoft Corp., Redmond, Wash.), was used to determine statistical significance *(25, 26).*

RESULTS AND DISCUSSION

Generation of cancers in mammals requires the mutation or deletion of oncogenes or tumor suppressor genes, resulting in a loss of heterozygosity at those allele locations. Mutation (point mutations or frameshift mutations) and deletion of genes can be induced by exposure of cells to genotoxic chemicals *(1, 6).* Many different short-term genetic

TABLE 2. *Induction of mutations in the* Salmonella *mutagenicity test, with or without exogenous metabolic activation (5% S9 fraction), by 2-dodecylcyclobutanone (2-DCB)*[a]

Strain	0% or 5% S9 fraction	2-DCB/HIS$^+$ colonies per well					Positive control
		0 mg	0.05 mg	0.10 mg	0.50 mg	1.00 mg	
TA98	0%	4.00 ± 0.50	3.83 ± 0.33	3.5 ± 0	3.67 ± 0.17	3.33 ± 0.88	111 ± 4.16
	5%	3.33 ± 0.44	2.33 ± 0.67	1.83 ± 0.33	2.00 ± 0.29	2.83 ± 0.17	92.2 ± 3.25
TA100	0%	16.2 ± 1.64	16.2 ± 1.30	19.2 ± 0.67	17.0 ± 0.76	16.8 ± 1.83	159 ± 8.26
	5%	13.2 ± 3.09	9.67 ± 1.59	16.2 ± 4.32	13.7 ± 1.17	14.2 ± 3.17	205 ± 4.49
TA1535	0%	3.50 ± 0.29	1.50 ± 0.29	2.17 ± 0.17	2.83 ± 0.17	3.50 ± 0.76	126 ± 4.07
	5%	2.16 ± 0.67	1.17 ± 0.44	1.50 ± 0.29	1.67 ± 0.73	1.33 ± 0.44	91.2 ± 7.91
TA1537	0%	2.00 ± 0.29	1.83 ± 0.44	2.00 ± 0.76	2.17 ± 0.17	1.33 ± 0.60	54.4 ± 5.84
	5%	1.17 ± 0.33	0.50 ± 0.29	1.67 ± 0.73	1.0 ± 0.29	1.50 ± 0.29	42.8 ± 1.59

[a] Results were tabulated from three independent experiments. There was no statistically significant increase in 2-DCB–induced mutations as determined by Students' *t* test ($n = 3$, $\alpha = 0.05$). Positive control compounds without metabolic activation were methyl methanesulfonate (TA100 and TA1535), 2-nitroflourene (TA98 and TA1537), and 2-aminoanthracene for all strains when metabolic activation was used.

toxicology tests have been used by regulatory agencies, the pharmaceutical and chemical industries, and academic researchers to predict a chemical's genotoxic potential, including the *Salmonella* mutagenicity test and the yeast DEL assay *(2, 15, 22, 26)*. There is a considerable body of research pertaining to the testing of food additives and food processing–created compounds in short-term genotoxicity assays. Mutagenic activity of thermally processed foods has been well established *(17, 28)*. A number of studies have confirmed the mutagenicity of cooked meats and their fats *(21, 32, 33)*. Unlike thermally processed meats and their fats, irradiated meats have previously tested negative in short-term genotoxicity tests *(30)*. In this work, the *Salmonella* mutagenicity test and the yeast DEL assay were used to assess 2-DCB's genotoxic potential.

Sommers *(25)* evaluated the potential genotoxicity of 2-DCB using the *Escherichia coli* tryptophan reverse mutation assay and obtained negative results using tester strains WP2 (pKM101) and WP2 *uvrA* (pKM101). In the *Salmonella* mutagenicity test, the strains TA98 and TA1537 are used to detect induction of frameshift mutations (addition or subtraction of nucleotides in the bacterial chromosome), whereas TA100 and TA1535 detect the generation of point mutations (Table 1). 2-DCB did not induce mutations in tester strains TA98, TA100, TA1535, and TA1537, with or without exogenous metabolic activation (5% S9 fraction) as determined by Student's *t* test ($n = 3$, $\alpha = 0.05$) (Table 2). Because the *Salmonella* mutagenicity test measures induction of frameshift mutations, in addition to point mutations, additional information as to 2-DCB's genotoxic potential, or lack of genotoxic potential, is provided over that of the *E. coli* tryptophan reverse mutation assay *(25)*. No effect on bacterial viability was observed by examination of the bacterial lawn in the top agar. Results for the negative control (solvent) and positive controls (120 μg per well of MMS, 10 μg per well of 2-NF, or 10 μg per well of 2-AA) were consistent with historical data *(2, 8, 15)*. The concentration of 1 mg per well of 2-DCB in the Miniscreen assay is the equivalent of the maximum allowed concentration (5 mg per plate) in the standard plate incorporation assay *(2, 8, 15)*.

In *S. cerevisiae*, intrachromosomal (DEL) recombination is inducible by both mutagenic and nonmutagenic carcinogens that cause DNA strand breaks, including those responsible for oxidative damage to DNA (benzene and benzene metabolites) and enzyme inhibition (hydroxyurea, p-benzoquinone) *(9, 22–24, 26)*. DEL recombination is also inducible genetically by mutation of DNA repair genes (*rad2*) and those involved in cell cycle maintenance (*rth1*) *(27)*. Because the yeast DEL assay only measures genomic rearrangement in viable cells, it does not produce false-positive results due to induction of nongenotoxic cell death *(11, 26)*. 2-DCB did not induce intrachromosomal recombination in the yeast DEL assay as determined by Student's

TABLE 3. *Induction of intrachromosomal (DEL) recombination in* Saccharomyces cerevisiae *RS112 by 2-dodecylcyclobutanone (2-DCB)*[a]

	2-DCB					MMS, 50 μg/ml
	0 mg/ml	0.63 mg/ml	1.25 mg/ml	2.5 mg/ml	5.0 mg/ml	
Recombination frequency	0.72×10^{-4}	0.97×10^{-4}	0.62×10^{-4}	1.01×10^{-4}	1.04×10^{-4}	16.7×10^{-4}
Standard error	0.15×10^{-4}	0.15×10^{-4}	0.18×10^{-4}	0.60×10^{-4}	0.67×10^{-4}	1.24×10^{-4}
Viability (%)	100	85.3	86.5	76.9	28.5	54.8

[a] Results were tabulated from three independent experiments. There was no statistically significant increase in 2-DCB–induced intrachromasomal recombination as determined by Student's *t* test ($n = 3$, $\alpha = 0.05$).

t test ($n = 3$, $\alpha = 0.05$), even when cell viability was reduced to 28.5% ($n = 3$, $\alpha = 0.05$) (Table 3).

These results are in agreement with those obtained previously using the *E. coli* tryptophan reverse mutation assay *(25)*. They are also in agreement with long-term multigenerational feeding studies in multiple rodent species (rats, mice, guinea pigs, and rabbits) that tested the toxicological safety (tumor formation, teratogenesis, and fertility) of irradiated meat products that contained 2-DCB *(30, 34)*. They are in contrast to studies in which 2-DCB was reported as a potential genotoxin, due to a weak response in the comet assay, which measures increases in DNA strand breakage via examination by gel electrophoreses as its end point *(13, 14)*. Because the comet assay is not validated for detection of weak genotoxins and can produce false-positive results when cell viability is reduced (nongenotoxic cell death) *(18, 31)*, the authors cautioned against misinterpretation of the results of those studies *(13, 14)*. A review of the reports by the European Commission Scientific Committee on Food and Health Canada did not support the claim of genotoxicity for 2-DCB *(16, 18)*. The results from the current work, which examined the ability of 2-DCB's to induce either mutations or chromosome rearrangements, indicate that previous equivocal results obtained using the comet assay may be due to nongenotoxic cell death as opposed to actual genotoxicity. Future work should include the testing of 2-DCB in mammalian cell lines or animals to assess its ability to induce mutations or chromosomal rearrangements.

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